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An accurate and transferable protocol for reproducible quantification of organic pollutants in human serum using direct isotope dilution mass spectrometry

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A robust method has been developed for easy transfer between analytical laboratories to obtain highly accurate and reproducible quantification of persistent organic pollutants (POPs) in micro-volumes of serum. This method is suited for analysts researching the impact of environmental exposure on human health. When performed by highly trained analysts, existing methods can produce high quality data; however, complex sample preparation steps often cannot be consistently replicated by laboratories, leading to variance in extraction recovery and quantitation. By combining stir-bar sorptive extraction (SBSE) with direct isotope dilution (D-ID) mass spectrometry quantification, a new analytical method was developed. The D-ID quantification significantly improved accuracy, corrected sample-to-sample irreproducibility, and reduced sample preparation time. Independent production of statistically identical data then confirmed transfer of the validated operating protocol to an off-site laboratory with different instrument models. SBSE performance was compared with industry-accepted extraction techniques. D-ID quantification was compared with peer-reviewed relative isotopic response factor (RF) quantification methods. Holding other variables constant, D-ID improved accuracy by 250% and precision by 300% compared with RF; SBSE improved accuracy by 37% compared to industry-accepted extraction methods. Limits of quantification of the analytes ranged from 60 pg g^{-1} to 1 μg g^{-1}. Protocol transfer exhibited <7% mean between-laboratory error and <2% mean within-laboratory RSD. These results indicate that a transferable method has been developed for academic, government, commercial, and clinical laboratories seeking to maximize throughput and improve quantitative validity. This validated method was applied in a recent clinical study to assess non-communicable disease in children in Pennsylvania, USA.

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Introduction

Emergent data has implicated environmental exposure, combined with genetics, as a potential causative factor in the development of certain disease states.[1–5] The US Centers for Disease Control and Prevention and the US National Institute for Occupational Safety and Health have endorsed the term "exposomics" to foster an increased understanding of how environmental exposure can interact with personal genetics, physiology, and epigenetics to impact overall health.[6] A link has been suggested between serum concentration of certain organic toxins and etiology of disease-states such as autism spectrum disorders, heart disease, diabetes, and lupus, among many others.[1,3,7–10] Taken collectively, these carbon-based toxins are known as persistent organic pollutants (POPs) and exhibit common characteristics such as semi-volatility, environmental persistence, low water solubility and inherent toxicity.[11] In 2001, 178 countries and the European Union signed the Stockholm Convention on POPs, which restricted or eliminated the production of certain chlorinated pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and chlorinated benzene compounds.[12,13] Recently, the World Health Organization developed guidelines for quantifying POPs in biological fluids to assess exposure.[14] Increasing interest has been placed in the development of methods to quantify POPs in serum for the purposes of improving human health, and disease diagnosis and prevention.[15,16] Understanding and defining the potential link between exposure to environmental contaminants, such as POPs, and the individualized health of human beings is the primary goal of exposomic research.[17,18]

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Exposomics is a rapidly advancing field that requires analytical methods to be accurately and reliably deployed in academic, government, clinical, and commercial laboratories. As the emergent field advances, studies of the human health impact of environmental toxins increasingly focus on chronic, not acute, POPs exposure (*e.g.* long-term ingestion and inhalation from environmental and industrial sources). Thus, regional and demographic data concerning human exposure to specific toxins has become increasingly important to researchers.[19]

Stir-bar sorptive extraction (SBSE) is a solvent-less alternative to traditional solid phase extraction (SPE)[20] that is rapidly gaining application in analytical laboratories. SBSE utilizes a glass-coated iron core wrapped in a polymeric extraction phase and functions by the same extraction mechanism as the industry-accepted solid phase microextraction (SPME).[21] In addition to a 50–100 fold increase in extraction phase volume compared with typical SPME,[22] SBSE also changes the extraction mechanism from a passive diffusion-based adsorption to active sampling by rapidly stirring an analytical sample.[23] Most SPE tools (*e.g.* cartridges, disks, filters, fibers) are incompatible with the analysis of biological fluids without significant sample preparation and cleanup. Therefore, most SBSE methods have arisen from research in water and agricultural matrices to avoid irreversible damage to the extraction-phase coating.[24] In contrast with other SPE methods which require a sample to be passed-through an extraction phase, the immersive capability and robust nature of SBSE bars allow cleaning to remove damaging agents, such as lipids and proteins.

For the extractions of analytes of mixed-characteristics, dual SBSE has typically been used as a tandem-in-time extraction.[25] For mixed volatility analytes, dual SBSE is conducted by immersive and headspace analysis simultaneously.[26] Following stir-bar extraction, analytes are thermally desorbed from the bar(s) in the inlet of a gas chromatograph (GC). The relative cost difference between sorbent stir-bars and other SPE tools means that for SBSE, more than other SPE methods, the efficient use of a limited number of stir-bars is often the limiting factor in overall processing time.

It has been shown that desorption of analytes from the stir-bar sorbent phase prior to analyte-extraction phase equilibrium yields non-reproducible results.[20] However, driving these extractions to equilibrium often requires prohibitively long extraction times; studies have shown that up to 14 hours is often required for equilibrium of POPs compounds.[27,28] Citing equilibration time constraints, studies have indicated the need to develop techniques to ensure pre-equilibrium sample-to-sample reproducibility in SBSE.[29] A true algorithmic quantification eliminates inaccuracy introduced by relative quantification methods like calibration curve and RF.[30] Accurate quantification is accomplished by isotope dilution mass spectrometry, as described by EPA method 6800.[31] EPA and peer reviewed methods using isotope dilution quantification for POPs require the analyst to generate calibration curves based upon relative isotopic RFs. All forms of calibration are relative by nature. True isotope dilution is a direct quantification that avoids the relative nature of external calibration curves or RFs. This type of quantification is called direct isotope dilution (D-ID).

Quantification by D-ID is based on spiking of known amounts of enriched isotopic analogs of each compound into a sample. Prior to extraction equilibration, equilibrium must be obtained between the endogenous and spiked isotopic compounds. Equilibration of the endogenous molecule with the spiked isotope in the sample alters the isotope ratio.[31] The altered isotopic ratio is essential for direct quantification. With the known isotopic abundance of both endogenous and spiked analytes, the amount of spike added to the known amount of sample, concentration of the spike added, and altered isotopic ratio, the concentration of the endogenous molecule in the sample can be directly calculated.[31] Being chemically identical, the endogenous and spiked isotopes are extracted with equivalent efficiency and recovery. This chemical resemblance is the distinct advantage of D-ID compared with calibration curve, internal standards, and RF quantifications. Utilized together, SBSE with thermal desorption introduces both molecular forms into the analytical method simultaneously.[31,32] This simultaneous thermal desorption ensures that both molecular forms experience identical environments throughout analysis. Once equilibration is achieved between endogenous and spiked compounds, D-ID is able to mathematically correct for many of the sources of error and variance associated with extraction, mass spectrometry, and relative quantification methods because the endogenous and isotopic forms are affected identically. These sources of error include, among others, imprecise sample preparation, poor extraction reproducibility, low analyte recovery, instrumental drift, sample loss, and physical or chemical interferences. Thus, D-ID reduces the contributions of random error and the analyst to overall quantitative quality, resulting in greater reliability and uniformity of accuracy and precision. This rapid and solid-phase equilibration process directly facilitates the ability to design an efficient and reproducible operating procedure.[32]

The primary goal of this research was to increase efficiency and reduce variance in sample preparation, while simultaneously improving quantitative accuracy without using external calibration curves or isotopic RFs. To increase efficiency, this research optimized single stir-bar SBSE to extract both volatile and nonvolatile analytes from a sample in a single extraction. To improve SBSE reproducibility and accuracy, an algorithmic correction was applied to reduce the influence of pre-extraction equilibrium analysis and imprecise sample preparation on quantitative quality. For the purpose of clinical application, sample volume was optimized to significantly reduce the required volume compared with existing methods. The second goal of this research was to demonstrate robustness and transferability of this analytical method among and between laboratories using automated gas chromatography-mass spectrometry (GC-MS) systems. Following demonstration of transfer, this validated method was applied to collaborative clinical research on non-communicable disease in Western Pennsylvania.

Results and discussion

Analyte recovery

Single and dual bar extraction recoveries were compared by replicate extractions of a prepared mixture of endogenous NIST-

traceable standards by each method. Total analyte recovery with single bar SBSE was comparable with dual bar SBSE, producing statistically identical recovery for 13 out of 15 analytes. As Fig. 1 demonstrates, the highest volatility analytes, benzene and toluene, presented recoveries 85% and 45% lower, respectively, in single step extraction compared with dual SBSE. Other POPs typically extracted using headspace methods: PCBs, PBDEs, phthalates, and pesticides displayed statistically identical recoveries using the single step analysis.

Single bar SBSE increased method efficiency and allowed twice as many samples to be extracted simultaneously, while producing recoveries comparable to dual SBSE. An additional benefit observed with single bar SBSE was the reduction of background siloxane peaks in the chromatogram. This was likely due to the reduction of the volume of PDMS inside of the TDU tube during desorption. Additionally, sorbent stir-bars possess an inherent lifespan. Manufacturer recommendations list this lifespan at 50-extractions; however, internal research determined that under light use (moderate pH and primarily aqueous samples), nearly 100 extraction could be performed before extraction efficiency fell below acceptable levels. The increasing popularity of SBSE stir-bars, combined with their relatively high cost, demands that laboratories make the most efficient use of the available number of bars. The improved efficiency afforded by extracting with one stir-bar instead of two would most obviously benefit laboratories that process large numbers of mixed-volatility samples.

Sample-to-sample reproducibility

Replicates of a prepared mixture of NIST-traceable endogenous standards were extracted from blood serum by single bar SBSE to determine sample-to-sample reproducibility. Prior to quantification, analysis was performed on the reagent serum to determine the concentration of any existing background contamination present in the serum. These background values were subtracted from any data obtained from the reagent serum, producing a so-called "blank-subtracted serum." Standards were added to the serum using an analytical balance, to enable quantification by mass, at concentrations within one order of magnitude of the respective LOQ for each analyte. Isotopic forms of each compound were spiked into each sample vial by mass. Precision was assessed as % RSD for comparative purposes. Unaltered peak areas were used to obtain "raw data" % RSD. This same data was then processed as a simple function of endogenous to spiked peak areas ($P_{endogenous}/P_{spike}$). As seen in Table 1, analyzing by raw peak areas produced mean total % RSD across all analytes of 11.9%. When the same data were processed by D-ID, mean total % RSD for all analytes improved to 4.2%.

Significant improvements in sample-to-sample reproducibility observed in the D-ID data were most reasonably achieved by compensating for analytical variance typically introduced to the sample preparation and quantification. For accurate quantification, the full D-ID equation from EPA Method 6800 was used for calculations.

Many peer reviewed and EPA methods still in use today define isotope dilution as an isotopic RF. Isotopic RF quantification relies on generating a calibration curve that plots RF (between an endogenous compound and its isotopic analog) *versus* concentration of endogenous standard.[33] This approach differs from D-ID, in which no calibration curves or RFs are generated. This way, D-ID eliminates the inexact nature of calibration curves. By decreasing variance, D-ID quantification

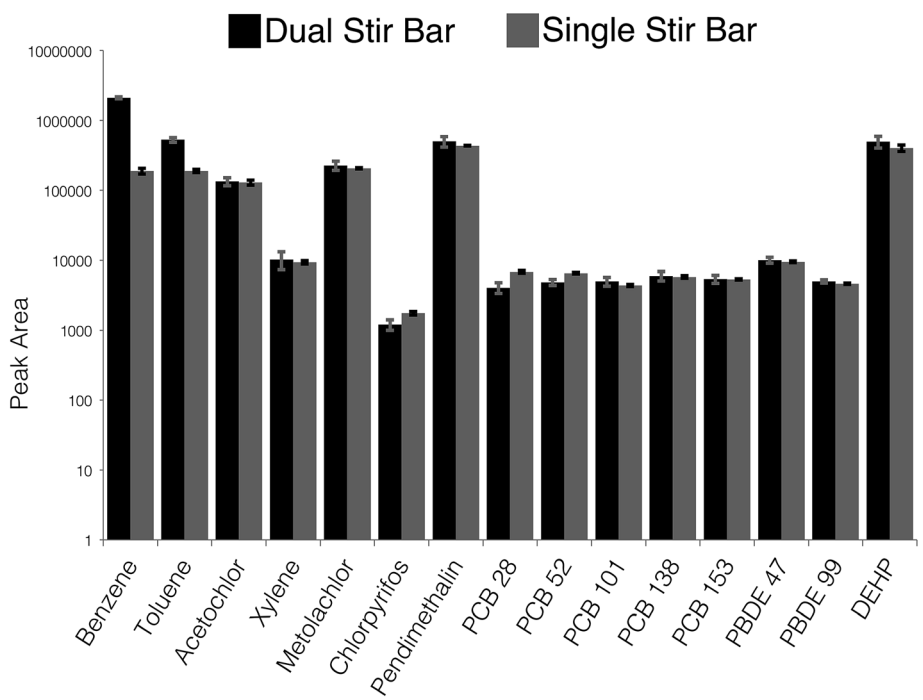

Fig. 1 A comparison of relative recovery between single and dual stir bar extraction, showing 95% CI ($n = 5$).

Table 1 A comparison of reproducibility of raw chromatographic peak areas with D-ID-corrected peak areas ($n = 5$)

Analyte	% RSD raw data	% RSD D-ID corrected
Benzene	29.7	9.36
Toluene	17.1	5.34
o-Xylene	13.5	5.67
PCB-28	13.9	4.49
PCB-52	10.6	2.28
PCB-101	7.26	3.58
PCB-138	9.21	4.08
PCB-153	10.4	2.01
PBDE-47	3.25	2.97
PBDE-99	5.95	1.42
Chlorpyrifos	9.84	5.66
Metolachlor	6.95	1.98
Acetochlor	19.3	8.45
Pendimethalin	10.1	0.812
DEHP	10.8	6.12

created confidence intervals that were narrower than those obtained by analysis of raw data, allowing for potentially actionable diagnostic results.

Comparison with existing methods

Single step extraction was compared with the traditionally accepted extraction method of SPME using both isotopic RF and D-ID mass spectrometry quantification of PCB-52 in 200 μL of blank-subtracted serum. Fig. 2 shows that SPME coupled with isotopic RF quantification produced inaccurate quantitative values with poor reproducibility. The addition of D-ID quantification to SPME improved accuracy from 24.6% to 9.66% error and precision from 16.9% to 5.61% within-run RSD. Single bar SBSE improved the accuracy over SPME from 9.66% to 6.07% error and increased the precision from 5.61% to 5.40% RSD. Single bar SBSE-D-IDMS significantly improved quantitative accuracy and precision, compared with industry-accepted SPME and calibration curves. A reasonable explanation for the improvements in SPME-D-ID over SPME-RF is the reduction by D-ID of many errors associated with sample preparation and analysis prior to extraction equilibrium.

Accuracy at limit of quantification

Data quality approaching a limit of quantification (LOQ) was assessed for both RF and D-ID quantification using PCB-52 and a quantification limit of 0.111 ng g^{-1}. Data was obtained by spiking isotopic and varying concentrations of endogenous PCB-52 into 200 μL of blank-subtracted serum and analyzing by single bar SBSE-D-ID. An isotopic RF calibration curve was generated for the endogenous compound at values 40% to 4000% above LOQ with $n = 5$ replicates at each point. This data was then processed by D-ID using data obtained from the same analyses. Fig. 3 shows that the RF data lost quantitative accuracy below 25 ng g^{-1} (two order of magnitude above the D-ID quantification limit) with a mean within-run % RSD of 10.6%. When the same data was processed by D-ID, all data points maintained quantitative accuracy with a mean within-run % RSD of 2.28%.

As suggested by theory, it was observed that calibration accuracy and precision worsened approaching the limit of quantification.[34] The calibration curve does not match the certified concentration below 25.0 ng g^{-1}. This work demonstrated the capability of D-ID to maintain quantitative accuracy, validity, and reliability approaching the instrumental LOQ.

Method validation

Concentrations of all analytes were experimentally determined in 200 μL of blank-subtracted serum and compared at the 95% CI against certified standards traceable to the National Institute of Standards and Technology (NIST). All quantified POPs were statistically identical to the NIST traceable certified concentrations, proving accurate quantification. A complete description of scientifically relevant chemical characteristics and figures of merit of the single bar SBSE-D-ID mass spectrometry method can be found in Table 2. All data were obtained from analyses of

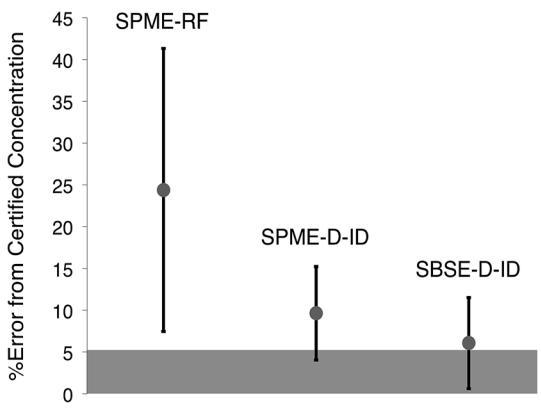

Fig. 2 A comparison of single bar SBSE and D-ID with existing, industry-accepted methods of extraction and isotopic quantification. The 95% CI ($n = 5$) is shown. Uncertainty of the calculated concentration is shaded.

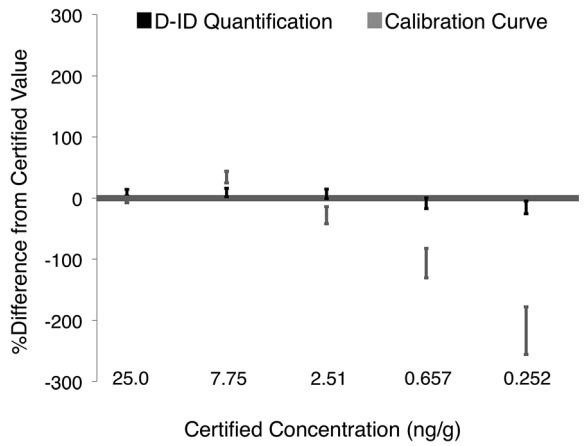

Fig. 3 A comparison of D-ID and RF quantifications approaching the PCB-52 quantification limit, showing 95% CI ($n = 5$). Uncertainty of the certified value (5%) is shaded.

samples containing endogenous and isotopic compounds spiked into blank-subtracted serum using an analytical balance. The calculated value represents the concentration of endogenous compounds present in the spiked serum prior to analysis calculated from the initial concentrations of NIST-traceable standards. Across all analytes, average accuracy exhibited 4.10% error with an average precision of 4.28% RSD.

This research specifically focused on the quantification of a selected group of environmental toxins. However, the mechanism of SBSE is governed by the octanol–water partition ($K_{o/w}$) of an analyte, extraction phase volume, and sample volume.[20] It is, therefore, reasonable to propose that, using identical sample volumes and stir-bars, this method could be expected to produce similarly high-quality data when expanded to analytes of $K_{o/w}$ values within the range of those included in this work (log $K_{o/w}$ 2.3–7.5). The validated method developed in this work was specifically optimized for the selected list of analytes; however, the mechanisms of extraction, separation, and quantification could allow a universal application to POPs of similar chemical characteristics.

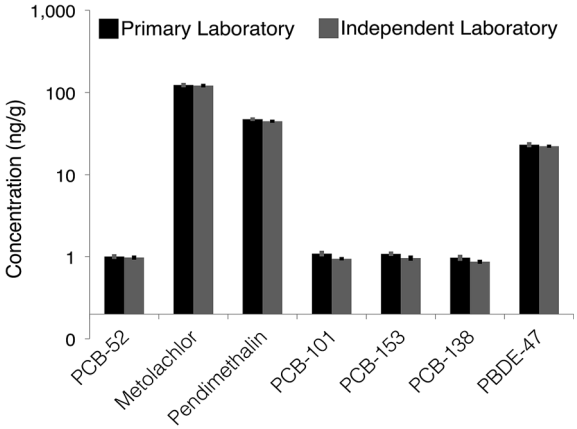

Fig. 4 Comparing results obtained by an independent laboratory with approximately two hours of training, with results obtained by primary laboratory, showing 95% CI ($n = 5$).

Between-laboratory method transfer

Transfer of method quality to an independent laboratory, as demonstrated in Fig. 4, produced results that were statistically comparable to the results obtained at the primary laboratory at the 95% CI. A chemically diverse suite of analytes was chosen to test robustness of the method transfer. Total within-laboratory reproducibility for the primary laboratory across all selected analytes was 2.52% RSD; the independent laboratory achieved total within-laboratory reproducibility of 1.32% RSD.

In commercial, clinical, and government laboratories, inherent biological variability is often compounded by poor analytical reproducibility introduced by necessary, but complex, sample preparation steps.[35] In these labs, emphasis has been placed on development of simple analytical procedures which use small volumes of blood serum to obtain high accuracy, actionable results.[36] Such analyses require methods that are statistically accurate, highly reproducible, and efficient. Highly complex sample preparation steps may be reproduced by expert analysts. But, by compensating for much of the potential variance introduced in sample preparation, this method has demonstrated the potential to be transferred between laboratories and analysts with approximately two hours of operational training.

Application to clinical research

The high accuracy and transfer capability of this optimized method are directly applicable to clinical chemistry, among other fields. As a proof of application, this section will detail analytical improvements in two recent IRB-approved collaborative studies with The Children's Institute of Pittsburgh (TCI) that investigated exposure to environmental toxins in children. In the first, existing methods using SPME were optimized for

Table 2 Figures of analytical merit comparing concentration values obtained by S-SBSE-D-ID ($n = 5$) in blank-subtracted serum with calculated concentrations. Unless noted, all concentrations are in units of μg g^{-1}

Analyte	log $K_{o/w}$[a]	log VP[a,b]	Calculated value	Experimental value[c]	|% Error|	% RSD	LOQ (ng g^{-1})
Benzene	2.3	7.12	9.14 ± 0.457	9.77 ± 1.12	6.89	9.36	163
Toluene	2.69	6.47	6.34 ± 0.317	5.93 ± 0.389	6.47	5.34	29.7
o-Xylene	3.12	6.03	11 ± 0.55	11.1 ± 0.774	0.908	5.67	6.41
PCB-28	5.62	0.731	1.33 ± 0.0665	1.27 ± 0.0701	4.51	4.49	2.46
PCB-52	5.84	0.731	1.33 ± 0.0665	1.29 ± 0.0362	3.01	2.28	0.111
PCB-101	7.07	0.731	1.33 ± 0.0665	1.28 ± 0.0563	3.76	3.58	0.159
PCB-138	6.83	0.731	1.33 ± 0.0665	1.29 ± 0.0647	3.01	4.08	0.309
PCB-153	6.68	0.731	1.33 ± 0.0665	1.27 ± 0.0314	4.51	2.01	1.26
PBDE-47	6.81	−0.602	0.531 ± 0.0265	0.503 ± 0.0183	5.27	2.97	1.49
PBDE-99	6.5	−0.602	3.27 ± 0.163	3.28 ± 0.0572	0.306	1.42	1.71
Chlorpyrifos	5.11	0.426	1.7 ± 0.085	1.64 ± 0.114	3.53	5.66	0.0648
Metolachlor	3.13	0.239	1.86 ± 0.093	1.8 ± 0.0438	3.23	1.98	0.193
Acetochlor	3.12	0.656	2.1 ± 0.105	2.25 ± 0.234	7.14	8.45	30.1
Pendimethalin	5.18	0.669	0.641 ± 0.0321	0.624 ± 0.00621	2.65	0.81	0.0621
DEHP	7.5	−1.08	0.61 ± 0.0301	0.562 ± 0.0746	7.87	6.12	1230

[a] Chemical values taken from material safety data sheets. [b] Vapor pressure. [c] Experimental values determined with $n = 5$ replicates showing 95% CI.

use with D-ID quantification. Multiple peer-reviewed sources claimed to improve sample-to-sample SPME reproducibility in blood serum.[37,38] However, these methods were unable to be replicated in this laboratory for serum-immersive SPME analysis. The analytical incompatibility of SPME with immersive extraction from complex matrices such as serum and plasma necessitated the development of new methods for the analysis of POPs in small volumes of serum. The second study used SBSE and D-ID together to quantify a suite of organic toxins. Comparing the average quantifiable concentration of toluene, *o*-xylene, PCB-138, and PCB-153, (toxins included in both studies) Fig. 5 demonstrates the improvement in LOQ afforded by single bar SBSE-D-ID (mean LOQ = 9.14 ng g^{-1}) over SPME-D-ID (mean LOQ = 57.5 ng g^{-1}), which allowed quantification of biologically relevant concentrations of POPs. Analyzing by SPME-D-IDMS only quantified the greatest outliers.

Table 3 shows a sample application of this method to non-disease state children in collaboration with TCI. Increased sample-to-sample reproducibility, improved mean accuracy and precision, and greater sensitivity over industry-accepted methods all combined to produce actionable medical findings by the collaborating physician.[19] Owing to improved sensitivity, sample volume was optimized to permit an appropriately high number of replicates to be performed on a relatively small sample of serum: five replicates were obtained from 1 mL of serum.

To obtain actionable results and discern variable changes from clinical analyses, high quality data must be obtained with narrow confidence intervals. National and regional laboratories are often equipped to produce similarly high quality data for large populations. However, in this age of increasingly personalized medicine, local academic, clinical, commercial, and government laboratories must be capable of generating the same highly reliable and reproducible data. Observed in Fig. 6, using metolachlor as an example, this method allowed for the identification of statistically outlying individuals when population and sub-populations showed no statistical deviation from national average.

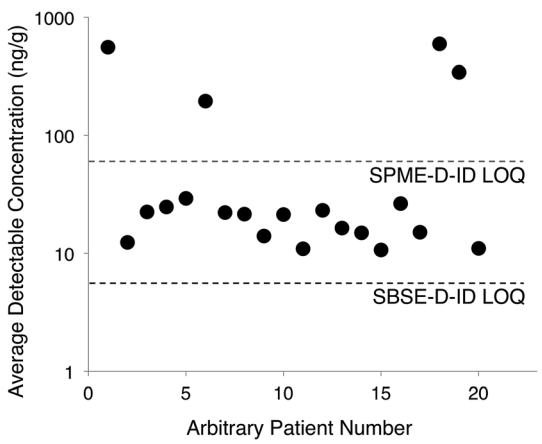

Fig. 5 Mean toxin concentrations obtained using SBSE-D-ID, comparing mean LOQ of SBSE-D-ID and SPME-D-ID for POPs quantified in both studies.

Table 3 Data demonstrating regional and local deviations of select demographics from national averages (n = 954, 615, 602, 481 national, respectively). Concentrations listed in units of ng g^{-1} serum whole weight. Male: $n = 4 \times 5$, female: $n = 5 \times 5$

	Toluene	PBDE-47	Chlorpyrifos
Males			
2–5 years	8.97	<1.49	0.157
6–9 years	9.91	2.12	0.173
Females			
2–5 years	16.4	2.3	0.179
6–9 years	71.4	<1.49	0.181
National mean[41]	0.114	26.8[a]	1.76[b]

[a] Concentration in units of ng g^{-1} of lipid. [b] Measured as the primary chlorpyrifos metabolite 3,5,6-trichlorpyridinol.

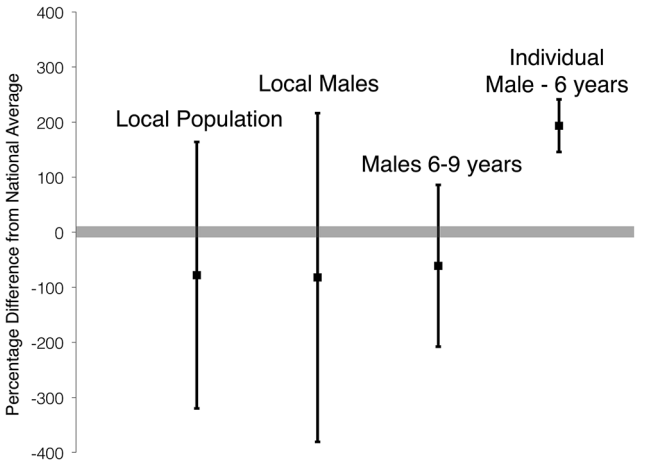

Fig. 6 Mean difference from national average of metolachlor in regional collaborative study, 95% CI shown ($n = 30 \times 5$, 27×5, 15×5, 5, respectively). Grey bar represents 95% confidence of national average.

The high throughput afforded by the increased efficiency and reduced sample preparation of this method enabled the generation of population and sub-population data for inter- and intra-comparison purposes. The reliability and sample-to-sample reproducibility of this method enabled high quality individualized analysis to be performed as well. The ability to reliably transfer among and between laboratories could allow local laboratories, with traditionally fewer resources than national laboratories, to generate the same high quality, reliable, and reproducible data.

Experimental

A standard containing 7 polychlorobiphenyl (PCB) congeners [2,4,4′-PCB (PCB-28), 2,2′,5,5′-PCB (PCB-52), 2,2′,4,5,5′-PCB (PCB-101), 2,2′,3,4,4′,5-PCB (PCB-138), 2,2′,4,4′,5,5′-PCB (PCB-153), 2,2′,3,4,4′,5,5′-PCB (PCB-180), and 2,2′,3,3′,4,4′,5,5′-PCB (PCB-209)] and standards for polybrominated diphenyl ether

(PBDE) congeners [2,2′,4,4′-BDE (PBDE-47) 1 and 2,2′,4,4′,5-BDE (PBDE-99)] were purchased from Sigma-Aldrich (St. Louis, MO). Benzene, toluene, *o*-xylene, (≥99% purity) and bis(2-ethylhexyl) phthalate (DEHP) were purchased from Fluka (St. Louis, MO). Certified standards for chlorpyrifos (99.5% pure), pendimethalin (98.8% pure), acetochlor (98% pure), metolachlor (98.6% pure), toluene-d_8, *o*-xylene-d_{10} were obtained from SPEX CertiPrep Group (Metuchen, NJ). A certified standard for benzene-d_6 was obtained from Cerilliant (Reston, VA). Chlorpyrifos-d_{10} (99% labeled), a standard mixture of PCB–$^{13}C_{12}$ (99% labeled), and standards for PBDE-47 and 99-$^{13}C_{12}$ (99% labeled) were obtained from Cambridge Isotope Laboratories, Incorporated (Tewksbury, MA). Pendimethalin-d_5 (98% labeled), acetochlor-d_{11} (98% labeled), metolachlor-d_6 (98% labeled), and bis(2-ethylhexyl) phthalate-d_{38} (DEHP-d_{38}) were obtained from C/D/N isotopes incorporated (Pointe-Claire, Quebec, Canada).

Extractions were carried out using 10 mm × 0.5 mm (length × film thickness) polydimethyl siloxane (PDMS) stir-bars, supplied by Gerstel (Mülheim a/d Ruhr, Germany) in 10 mL headspace vials for extractions (Sigma-Aldrich). Stirring was conducted using a 20-position magnetic stir-plate (Gerstel). A SPME assembly was purchased with a PDMS fiber of 30 μm film thickness (Sigma-Aldrich) for method comparison studies. Prior to use, the stir-bars were conditioned per manufacturer instructions. Thermal conditioning took place in a thermal conditioning unit (TCU) at 290 °C for 4 hours with a helium flow of 50 mL min^{-1}. HPLC-grade methanol (Sigma-Aldrich) and ultrapure (18 Ω) water were used for reagent dilution, glassware cleaning, and stir-bar cleaning.

Analyte selection

For development and optimization, previous studies guided the choices of the most representative analytes to encompass the many classes of chemical activity defined as POPs. Traditional POPs classes were included (PCB, PBDE), along with compounds that have not traditionally been classified as POPs but display the characteristics of semi-volatility, environmental persistence, and hydrophobicity.[11] Many of the analytes chosen for this research are not present on the Stockholm Convention list of POPs; this work was designed to include both banned POPs and products with similar chemical characteristics which have become environmentally ubiquitous.[39,40] Selected compounds ranged several orders of magnitude in hydrophobicity (octanol–water partitioning coefficient, $K_{o/w}$) and volatility (Torr). Three volatile organic compounds (benzene, toluene, and *o*-xylene), five PCBs (standard congeners 28, 52, 101, 138, and 153), two PBDEs (standard congeners 47 and 99), two organochlorine pesticides (metolachlor and acetochlor), one dinitroanaline pesticide (pendimethalin), one organophosphate pesticide (chlorpyrifos), and one phthalate (bis(2-ethylhexyl) phthalate) were chosen for this research.

Blood sample processing

In an International Standards Organization Class 5 cleanroom, whole blood samples obtained in an internal review board-approved collaboration with The Children's Institute of Pittsburgh (Pittsburgh, PA) were separated into red blood cells and serum using a calibrated centrifuge. Both serum and red blood cells were immediately transferred to airtight polyethylene containers and stored in the dark at −80 °C.

Stir-bar extractions

Prior to extraction, serum samples were brought to room temperature and approximately 200 μL was added by mass to a 10 mL extraction vial using an analytical balance. A mixture of enriched isotopic analogs was prepared by mass at a concentration equal to the certified concentration of the target analyte and spiked by mass into each sample vial. For this study, 100 μL of a mixture composed of ∼25 μg g^{-1} benzene, toluene, *o*-xylene; ∼4 μg g^{-1} PCB-28, PCB-52, PCB-101, PCB-138, PCB-153; ∼5 μg g^{-1} PBDE-47, PBDE-99, chlorpyrifos, metolachlor, acetochlor, pendimethalin, and DEHP was accurately spiked into each vial of serum.

For dual SBSE, one bar was added to the vial with 2 mL of ultra-pure water and one bar was hung in the headspace on a metallic wire with string. For S-SBSE, one stir-bar was immersed in the solution and ultra-pure water was added such that the extraction vial was completely filled to eliminate as much headspace as possible. Methanol was added, before extraction, to each sample to achieve a final methanol concentration of 20% (including the methanol from the isotope mixture) after dilution. Teflon-lined screw caps were fixed to the extraction vessels. Extraction was conducted at 1500 rpm for 60 minutes. The stir-bars were removed from the sample with tweezers and thoroughly rinsed with ultrapure water and dried with a lint-free tissue and deposited into a glass thermal desorption tube.

Desorption and chromatography

Desorption tubes were loaded into a tray and introduced sequentially into the thermal desorption unit (TDU) (Gerstel). The GC inlet was set to use programmed-temperature vaporization (PTV) on a chilled injector system (CIS-6) (Gerstel) inlet containing a CIS/TDU inlet liner packed with Tenax TA™ (Buchem B. V., Apeldoorn, The Netherlands). The CIS-6 injector was installed in an Agilent 6890 GC-5975 MS system (Agilent Technologies, Santa Clara, CA, USA). The sample loading and handling was performed by a dual-head robotic multipurpose sampling system (MPS-2) (Gerstel). The method parameters were programmed to the final desorption temperature of 280 °C and the analytes were desorbed under helium in the TDU before cryofocusing at −70 °C in the PTV system with liquid nitrogen. Finally, the CIS system was ballistically heated at 720 °C min^{-1} to 280 °C to transfer the analytes to the GC-MS for analysis. The analytes were separated chromatographically using an HP-5 MS column (30 m × 0.25 mm I.D. 0.25 μm film thickness, 5%-phenyl polydimethyl siloxane) at a 1.0 mL min^{-1} carrier gas flow rate. The GC oven was heated from 45 °C to 280 °C at 12 °C per minute, where it was held for 15 minutes. Ionization was conducted in electron ionization mode and mass selection/detection was accomplished in select ion mode (SIM) programmed to the quantitative and secondary ions selected for each analyte in the method development stage.

Quantification and statistics

For D-ID quantification, the mass spectrometer was operated in the SIM mode set to the quantification and confirmation ions (m/z) for each compound, which have been previously determined for each analyte and are available in peer-reviewed methods. During the development phase, means and standard deviations were calculated using five replicate analyses. The lower instrument limit of quantitative measurement (LOQ) was calculated as being equal to ten times the standard deviation of repetitive measurements on a blank, or $10s_{bl}$.[35] Precision was evaluated as percent relative standard deviation (% RSD) and 95% CI were determined for comparison with certified concentrations. Accuracy was determined by calculation of percent error from a certified value. The fragmentation of the enriched compound was studied to ensure that the quantification ion of the enriched analyte lay in the same fragmentation pathway as the quantification ion of the target analyte. Validation was performed by spiking certified standards for each compound into blank-subtracted blood serum and analyzing by the above method. Quantitative experimental results were compared against certified values using five-replicate means and 95% CI.

Selected m/z data was automatically exported using MassHunter software and quantification was accomplished using known isotopic abundance, isotopic enrichment purity of spike, the amount of spike added to each sample, concentration of spike added, and the isotopic ratios in the spiked sample. The concentration of the unknown natural molecule was calculated as:

$$C_x = (C_s W_s / W_x) \times ({}^{i}P_s - (R_{i/n} \times {}^{n}P_s)/(R_{i/n} \times {}^{n}P_x) - {}^{i}P_x)$$

$$R_{i/n} = \text{peak area of isotopically enriched molecule/peak area unenriched molecule}$$

In the D-ID quantification procedure,[31] C_s and C_x are the concentrations, in μmole g^{-1}, of the selected analyte in the isotope-enriched spike and the spiked sample, respectively. ${}^{i}P_s$ and ${}^{i}P_x$ are the percent purity of the isotopically enriched molecule in the spike and the pre-spiked sample, respectively. Likewise, ${}^{n}P_s$ and ${}^{n}P_x$ are the percent purity of the naturally occurring analyte in the spike and the pre-spiked sample, respectively. Finally, W_s and W_x are the masses of the spike and sample, respectively.

Quality reproduction

Unenriched versions of one organochlorine pesticide, one dinitroanaline pesticide, one tetra-substituted PCB, two co-eluting hexa-substituted PCBs, and one PBDE were spiked into a mixture of ultrapure water. Five replicates were processed at an off-site, independent laboratory by an analyst that received minimal training (*i.e.*, two hours) on the extraction, analysis, and quantification steps. Accompanying the reagents and supplies were electronic versions of the analytical protocol, instrumental and data processing methods, and quantification and automation software developed in this laboratory. Samples were processed by the independent analyst and final quantitative values were generated automatically and sent back to the primary laboratory for comparison.

Conclusions

This optimized method was the first to combine solid phase extraction of environmental organic toxins with true D-ID quantification. A novel workflow was also developed, taking advantage of the ability of D-ID to compensate for analytical variance, to transfer this method to an off-site laboratory with approximately two hours of training. D-ID mass spectrometry was shown to significantly improve quantitative accuracy and reproducibility when applied to both SPME and SBSE and single-bar SBSE improved efficiency over traditional dual-bar SBSE. Clinical application of this method produced actionable and individualized data for medical researchers. The cost effectiveness, sample-to-sample reproducibility, and transferability of this method could enable localized medical and environmental researchers to achieve data quality similar to national laboratories but on an individualized scale. Sample volume was reduced to 200 μL, a significant improvement over many existing methods. This method is attractive for laboratories seeking to maximize throughput while simultaneously achieving accurate quantification to improve validity and fidelity of results. Ongoing research for this laboratory involves collaborating on projects to include additional sample types and POPs classes to implement a reliable, repeatable, and robust D-ID enabled quantitative method using SBSE-GC-MS.

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